Cell Stem Cell
Minireview

DNA Demethylation in Pluripotency and Reprogramming: The Role of Tet Proteins and Cell Division

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Cytosine methylation is found in the genomes of many plants and animals and has been associated with transcriptional silencing in mammals. At critical stages in embryo development, when cellular potential is reset, DNA methylation is lost in a series of "sequential waves." The mechanism underlying this is controversial and complex. Several new reports now suggest that TET enzymes and cell division are important for these in vivo transitions as well as for experimentally induced reprogramming.

Introduction

In mammalian cells, DNA methylation occurs exclusively at the C5 position of cytosine, predominantly in the context of CpG dinucleotides. It is generated and maintained by two general classes of enzymes: the de novo methyltransferases, such as DNMT3a and 3b, which set up DNA methylation patterns in early development, and the maintenance methyltransferases, such as DNMT1, which copy those patterns onto daughter strands during DNA replication as cells divide. Because 5-methylcytosine (5-mC) is a rather stable epigenetic modification, cytosine methylation was initially viewed as being essentially unidirectional and this has prompted some heated discussions about how DNA methylation could be erased in vivo and whether DNA replication is required for erasure or not (Wu and Zhang, 2010). In principle, active (replication-independent) DNA demethylation could be achieved by removal of the the methyl group, by removal of the base itself, or by conversion of the base into an intermediate that could be resolved or replaced by unmodified cytosine. In contrast to this scenario, cytosine methylation would be progressively lost in dividing cells by passive dilution if, for example, the maintenance methyltransferase DNMT1 or its cofactor UHRF1 are absent or somehow diverted. The discovery that TET enzymes can catalyze the conversion of 5-mC to 5-hmC in vivo (Figure 1, upper panel) (reviewed in Pastor et al., 2013) and that 5-hmC is no longer recognized by UHRF1 (Hashimoto et al., 2012) suggests that replication-dependent and -independent processes could combine to bolster the regulated loss of methylcytosine from the genome. In this Minireview we have highlighted three recent reports (Blaschke et al., 2013; Ficz et al., 2013; Habibi et al., 2013) that, together with some additional studies (Leitch et al., 2013; Yin et al., 2013), have shown that ESCs grown in the presence of Erk1/2 and Gsk3β inhibitors (so-called 2i conditions) achieve a "naive state" in which the genome becomes hypomethylated (and reminiscent of early blastomeres seen in vivo) by potentiating Tet-mediated conversion of 5-mC to 5-hmC while simultaneously curtailing methyltransferase activities.

Chasing the Swell: Sequential Waves of Global DNA Demethylation in the Developing Embryo

Two waves of DNA demethylation have been described that globally reset identity in the developing mammalian embryo (Figure 1, lower panel). The first wave occurs soon after fertilization and gives rise to the totipotent zygote and subsequently to the formation of pluripotent cells within the inner cell mass (ICM). A second wave of reprogramming takes place later, as Primordial Germ Cells (PGCs) develop and migrate toward the genital ridges. These germ cells and their progeny will develop into the haploid gametes that are required to establish the next generation. The specialized gametes display several distinctive epigenetic and structural traits, such as the replacement of histones by the arginine-rich nuclear protamines, which must be replaced or erased shortly after fertilization to allow the zygote to undergo transcriptional activation and regain pluripotency. The extensive remodeling that is required to reset each parental genome is not symmetrical; the paternal pronucleus undergoes active DNA demethylation, while methylation is lost more gradually from the maternal pronucleus through cell division and cleavage (Santos et al., 2002). Loss of CpG methylation from the paternal genome is achieved by TET3-mediated oxidation (Gu et al., 2011; Iqbal et al., 2011; Wossidlo et al., 2011), while the maternal pronucleus remains protected from TET3 activity by PGC7 (Nakamura et al., 2012), and both are reliant upon passive DNA demethylation achieved through inefficient maintenance (Rougier et al., 1998, Gu et al., 2011). Oxidation of 5-hmC to 5-formylcytosine (5-fC) and 5-carboxymethylcytosine (5-caC) may also contribute to replication-independent DNA demethylation, and a significant role for DNA repair in reinstating an unmodified CpG state has also been proposed (Inoue et al., 2011; Hajkova et al., 2010; Wossidlo et al., 2010).

Once the pluripotent ICM is established, there is extensive de novo methylation as the embryonic lineages are specified. A subset of cells within the epiblast, from which the PGCs arise, undergo a second wave of reprogramming in which this newly established methylation is erased and germ cell specific genes are reactivated. Recent studies concentrating on the timing of

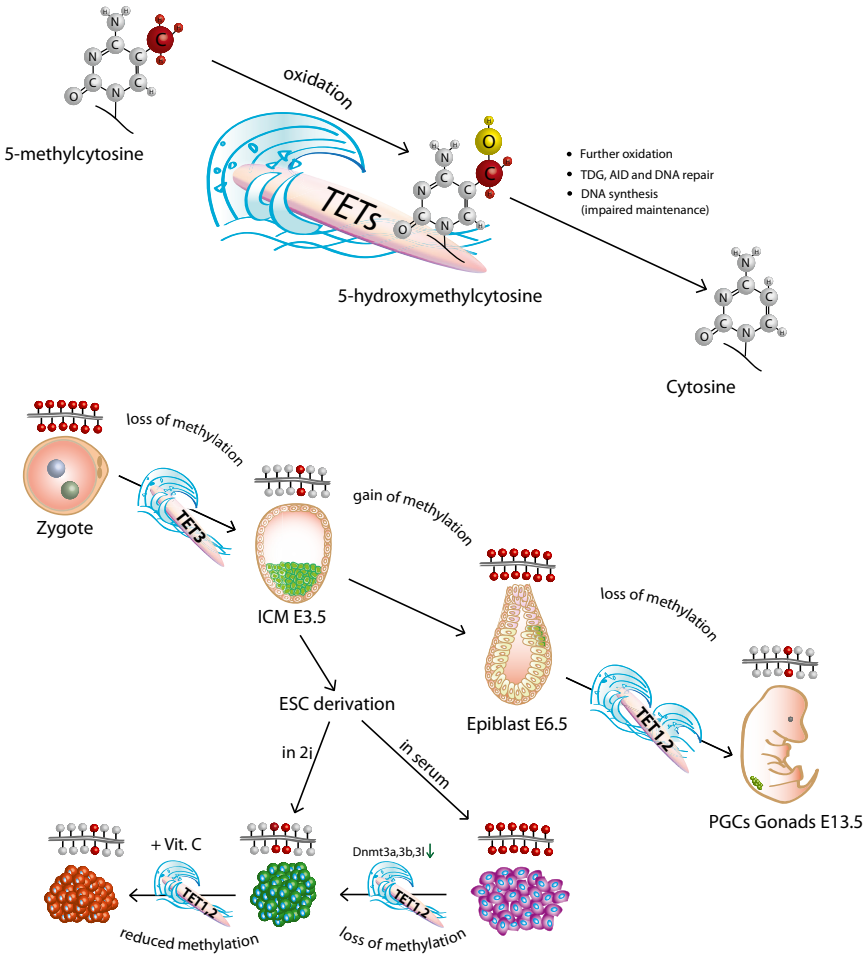

Figure 1. The Role of Tet Proteins in DNA Demethylation

The upper panel depicts the Tet-mediated oxidation of 5mC (upper) to generate 5hmC (middle), which is subsequently converted, by different agents and processes, to unmodified cytosine (lower). The methyl group is shown in red and the hydroxyl group is shown in yellow. The lower panel depicts the DNA methylation status of tissues at progressive stages of early mouse development from E0 to E13.5. Transitions where Tet proteins are critical are highlighted (surf board) and waves of DNA methylation and demethylation are indicated. Global methylation profiles of ESCs derived from the ICM, and grown in serum plus LIF, 2i, or 2i plus vitamin C are shown, in which increased hypomethylation is evident.

DNA methylation loss in mouse PGCs indicate a strict temporal order of events; demethylation begins as PGCs are specified and targets the promoters of a wide range of genes including markers of pluripotency and germ cells, and loss of methylation at Imprinted Control Regions (ICRs) occurs later, with LINE repeats and X-linked loci resisting demethylation until cells enter the gonads (Guibert et al., 2012; Seisenberger et al., 2012). In vivo BrdU incorporation studies have revealed that PGCs divide between E9.5 and E12.5, with a calculated division rate of once every 12.6 hr (Kagiwada et al., 2013). This, together with the downregulation of *Dnmt3a*, *3b*, *3l*, and *Uhrf1*, may provide an opportunity for passive demethylation during the first few days of PGC migration (Kagiwada et al., 2013; Seisenberger et al., 2012). PGCs express maximal Tet1 and Tet2 levels between E9.5 and E10.5 (potentially converting 5-mC to 5-hmC globally), while BER components are prominent only at later stages, but both may contribute to the removal of 5-mC at different regions of the genome (Hackett et al., 2013; Hajkova et al., 2010; Kagiwada et al., 2013).

Global Hypomethylation of ESCs Is Induced by 2i and Generates a Naive but Immortal State

Examining the biology of reprogramming in vivo has been challenging because it requires access to a very small number of embryonic cells that are in constant flux (Hackett et al., 2013; Santos et al., 2002; Smith et al., 2012). Embryonic stem cells (ESCs) that are derived from the preimplantation blastocyst have been widely used as immortal surrogates to represent the pluripotent epiblast and ICM (Nichols and Smith, 2009). ESCs grown or isolated in serum supplemented with leukemia inhibitory factor (LIF) are, however, surprisingly heterogeneous, as exemplified by *Nanog* expression (Chambers et al., 2007), and are primed to differentiate. Growth in media supplemented with two small-molecule kinase inhibitors (2i) promotes the establishment of a ground state of pluripotency (Ying et al., 2008) in which cells are much less heterogeneous, and several recent studies have begun to unravel the epigenetic basis of this ground state (Leitch et al., 2013; Blaschke et al., 2013; Ficz et al., 2013; Habibi et al., 2013). Earlier this year, Leitch, Hajkova, and collaborators (Leitch et al., 2013) demonstrated that several ESC lines isolated under 2i conditions (ES-2i) were hypomethyated as compared to those derived in conventional serum-based media (ES-serum) (Figure 1, lower panel, left). Loss of methylation was apparent at CpG islands and included LINE1 elements and minor and major satellite sequences, while IAPs and differentially methylated regions (DMRs) of imprinted genes resisted demethylation. A shift in methylation profiles was evident as early as five passages after 2i addition, and the authors tracked this down to a rapid decline of *Dnmt3a*, *3b*, and *3l* genes in 2i conditions that was in part mediated by PRDM14 repressing *Dnmt3b* (Leitch et al., 2013). New data from the Reik and Stunnenberg laboratories not only confirm and expand these analyses, but they also suggest that 2i enhances TET1/2 activity (and consequently 5-hmC levels), thereby providing an explanation for impaired maintenance methylation and "passive" loss of DNA methylation seen in ESCs switched to 2i conditions (Ficz et al., 2013; Habibi et al., 2013). Both new studies showed that methylcytosine was lost from gene bodies, non-CGI and CGI promoters, SINE and LINE1 elements, and enhancers and bivalent loci upon 2i

switching (albeit with slightly different timings), while IAPs and ICRs remained protected. Habibi et al. showed that regions that maintained DNA methylation correlated with those marked by H3K9me3 both in serum and in 2i, while TET1 target loci were particularly sensitive to DNA demethylation (Habibi et al., 2013). To understand how these findings relate to the waves of reprogramming that occur in vivo, these global methylation data sets were compared to those obtained from different stages of ICM and PGC development. This comparison revealed a close correspondence between ESCs grown in 2i and ex vivo cells from the mouse ICM at E3.5, or migratory PGCs at E9.5. In contrast, the high levels of 5-mC detected in ESCs grown in serum plus LIF resembled the methylome of cells isolated from the postimplantation mouse epiblast at around E6.5 (Ficz et al., 2013; Habibi et al., 2013) (Figure 1, lower panel).

Tet-Mediated Conversion of 5-mC to 5-hmC Is Enhanced by Vitamin C

Vitamin C, or ascorbate, has recently emerged as a factor facilitating DNA demethylation. It is a necessary supplement in human embryonic stem cell (hESC) media because cells stop proliferating two or three passages after its removal and require higher concentrations of b-FGF to be maintained (Chung et al., 2010). Vitamin C regulates the demethylation of promoter regions of more than 1,800 genes in hESCs, where it is associated with increased expression of a subset of these genes, which are implicated in cellular growth, proliferation, and pluripotency. In iPSC reprogramming, vitamin C is thought to overcome the senescence block and facilitate the pre-iPSC transition (Esteban et al., 2010), enhance the activity of H3K36 demethylases (Wang et al., 2011), and prevent imprinted *Dlk1-Dio3* erasure (Stadtfeld et al., 2012), all of which are important for increasing reprogramming efficacy. New studies from the laboratories of Ramalho-Santos, Wang, and Wang reveal a central role for TET proteins in this process (Blaschke et al., 2013, Minor et al., 2013; Yin et al., 2013). Treatment of mouse embryonic fibroblasts with vitamin C has been reported to result in a rapid increase (within 1 hr) in the abundance of 5-hmC, without any apparent change in Tet levels (Minor et al., 2013). Vitamin C can interact with the catalytic domains of TET1 and TET2 and enhance their enzymatic activity by altering structure and helping with product recycling. Mouse ESCs contain high levels of TET1 and TET2 and vitamin C addition is reported to result in a substantial and rapid increase in the level of genome-wide 5-hmC (Blaschke et al., 2013; Yin et al., 2013). Yin and colleagues have shown that global 5-mC levels in ESCs fell by 40% within 3 days of treatment and that this decline was dependent on TET1/TET2 (Yin et al., 2013). Blaschke et al. demonstrated that mouse ESCs grown in 2i progressively lose 5-mC at the promoters of previously methylated genes 12–72 hr after vitamin C addition (Blaschke et al., 2013). Because repetitive elements such as IAPs and ICRs gain 5-hmC upon vitamin C treatment, but remain protected from subsequent demethylation, the authors have suggested that this epigenetic profile resembles that of the in vivo early blastocyst and claim that (irrespective of 2i) without vitamin C, mouse ESCs acquire an epiblast-like methylation profile.

Harnessing the Waves: A Role for Tet Proteins and Cell Division in Experimental Reprogramming

Cells progressively acquire stable lineage-specific patterns of DNA methylation as they differentiate. This inherited epigenetic memory is difficult to fully erase by standard experimental reprogramming approaches (Bock et al., 2011). A better understanding of how DNA demethylation is achieved in vivo and how lineage identity is properly reset might be hugely informative for streamlining the transdifferentiation and dedifferentiation reprogramming strategies currently being developed for therapeutic purposes. In this regard, several recent studies have focused on the likely contribution of TET proteins to specific reprogramming scenarios (Doege et al., 2012; Costa et al., 2013; Gao et al., 2013; Piccolo et al., 2013; Vincent et al., 2013). Doege et al. have shown that the overexpression of *Oct4*, *Sox2*, *Klf4*, and *c-Myc* (OSKM) in mouse embryonic fibroblasts (MEFs) activated *Tet2* expression (*Tet1* and *Tet3* levels remained unchanged) and induced an increase in 5-hmC levels globally, as determined by immunofluorescence. Increased hydroxylation was evident at various pluripotent loci such as *Nanog* and *Esrrb* (Doege et al., 2012), implicating TET2-mediated hydroxylation in pluripotent reprogramming. Consistent with this idea, TET2-depleted MEFs were unable to generate OSKM-induced iPSC colonies. From this, the authors have argued that 5-hmC, rather than being an intermediate in 5-mC demethylation, could act as an independent epigenetic modification (Doege et al., 2012). In a related study in which the physical interaction between TET proteins and NANOG was described (Costa et al., 2013), overexpression of both of these factors was shown to enhance reprogramming while depletion of Tet1 reduced iPSC formation by MEFs. TET1 and NANOG were envisaged to act together and enhance the hydroxylation of loci such as *Esrrb* and *Oct4*, priming these genes for both activation and demethylation. Because TET2 can also physically associate with NANOG, TET2 overexpression, like TET1 overexpression, increased the efficiency of iPSC formation. The extent of the overlap between TET1 and TET2 target genes and the extent to which these endogenous proteins can compensate each other, however, remain largely unknown.

A recent reprogramming study has confirmed that TET1-mediated hydroxylation of the *Oct4* gene is accompanied by eventual demethylation, changes in histone modifications, and changes in gene reactivation, and it has shown that TET1 can substitute for OCT4 in an OSKM reprogramming cocktail (Gao et al., 2013). This substitution generated cells with lower 5-mC and higher 5-hmC than conventional iPSCs, which the authors have speculated might be significant in reducing the tumorigenic potential of this important cell type. Our laboratory has also shown, using a cell fusion-based system, that TET1 and TET2 are important for different aspects of pluripotent reprogramming (Piccolo et al., 2013). The study showed that Embryonic Germ Cells (EGCs) derived from late PGCs were able to induce imprint erasure from the genome of a somatic cell fusion partner and that this was critically dependent on TET1. Although the somatic genome accumulated 5-hmC at several ICRs within 2–3 days of fusion to EGCs, complete reversion to unmodified CpGs was protracted and required multiple rounds of cell division, similar to the dynamics of PGC development seen in vivo (Piccolo et al., 2013). Pluripotent reprogramming induced by

fusing somatic cells with ESCs was also associated with a rapid accumulation of 5-hmC at pluripotency loci within the somatic genome (such as Oct4), a process that may require Tet2, but is critically dependent upon DNA synthesis within the newly formed heterokaryons (Tsubouchi et al., 2013; Foshay et al., 2012; Piccolo et al., 2013). In another recent study in which ESCs were used to generate induced PGCs (iPGCs) expressing Blimp1 and Dpp3, genome-wide demethylation was shown to occur in two successive stages, somewhat reminiscent of PGC development in vivo. iPGCs generated from Tet2 null ESCs depleted of Tet1 showed a global reduction in 5-mC across the genome, but remained hypermethylated at specific loci (Vincent et al., 2013). The authors have interpreted this to mean that Tet1, Tet2, and 5-hmC may be dispensable for initial steps in DNA demethylation but are required to enable the re-expression of specific germ-cell-associated genes. This observation underscores the importance of impaired methylation maintenance, coupled to cell division, as a likely mechanism for generating genome-scale loss of 5-mC in vitro.

Conclusions

ESCs that lack both de novo and maintenance methyltransferase activities show a predictable lack of 5-mC but remain pluripotent, as judged by alkaline phosphatase activity and expression of a variety of pluipotency-associated genes (Tsumura et al., 2006). Likewise, ESCs lacking DNMT1, DNMT3a, and DNMT3b can give rise to pluripotent cells upon nuclear transfer, but the resultant cells contribute mostly to the extraembryonic tissues (Sakaue et al., 2010). These data suggest that although embryonic cell lines are relatively tolerant to an absence of DNA methylation, execution of their pluripotent status is difficult in the absence of stable gene silencing. Consistent with this idea, a recent study has shown that although ESCs lacking DNMT1, DNMT3a, and DNMT3b can differentiate to generate embryoid bodies in vitro, they fail to silence Oct4 completely and were prone to revert to an undifferentiated state (Schmidt et al., 2012). These studies, when taken together, emphasize that DNA methylation is not required to establish or maintain pluripotency, but it is vital in securing the stable transition toward differentiated progeny.

In this Minireview we have tried to capture some of the most recent developments within a fast- moving field in which the roles of TET proteins and cell division in DNA demethylation during experimental and "natural" reprogramming are being scrutinized. Evidence from a range of different in vitro reprogramming studies has suggested that TET proteins and/or replication-dependent (passive demethylation) processes probably underlie the conversion to an ESC-like or PGC-like state. Deletion of *Tet1* or *Tet2* in mice was initially shown to have little effect on their fertility or methylome, but these reports have given way to new studies showing a predictable increase in 5-mC levels in the absence of both *Tet1* and *Tet2* (Dawlaty et al., 2013). Whether some of the differences between in-vitro- and in-vivo-based studies reflect compensation by maternally derived Tet proteins, by Tet3, or by other demethylating factors remains unclear. However, these possibilities highlight the need to investigate redundancy between Tet family members and discover the mechanisms that recruit specific Tet proteins to particular subsets of genes. A more fundamental uncertainty in the field focuses on whether 5-hmC represents an intermediate in cytosine demethylation, or alternatively, represents an independent epigenetic mark that delineates loci that are potentially active or that are about to be expressed.

We have highlighted three new papers that show that ESCs grown in circumstances where FGF and Erk signaling is inhibited, and supplemented with vitamin C, become hypomethylated and display a methylome that resembles that of cells within the ICM or of PGCs. All of these studies, as well as some prior reports, have implicated TET proteins and 5-hmC in these transitions. As well as providing a plausible explanation for the perplexingly high global levels of cytosine methylation detected in conventionally grown ESCs, these studies have begun to exploit new genome-scale technologies that will aid our understanding of how signaling can fundamentally reset and reprogram the genome. While considerable debate remains regarding the relative importance of specific factors and processes in achieving this transition, it is likely that an intimate understanding of how global waves of demethylation shape the developing embryo will be important for improving efficacy and safety in experimental reprogramming. As an increasing number of laboratories strive to better understand and interrogate these waves of DNA demethylation, it is sobering to remember that surfing (or *he'e nalu*) is an ancient pastime, often described as one of the purest sports where the rider engages directly with an ever-changing environment. With this in mind, and with so much still to discover, there may never have been a better time to fashion your board and take to the water.

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